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zygote

<biology, genetics> A single diploid cell resulting from the fusion of male and female gametes at fertilization (sperm and ovum).

(11 May 1997)

Previous: zygonema, zygopodium, zygosis, zygosity, zygosperm, zygosphene, zygospore
Next: zygote intra-fallopian transfer, zygotene, zygotic, zygotic effect gene

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Vol. 11 8/13

From: Wilson, Michael
Sent: Tuesday, August 12, 2003 5:58 PM
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Kochav 1980, Dev. Biol. Vol. 79, pg 296-308.

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DEVELOPMENTAL BIOLOGY 79, 296–308 (1980)

From Cleavage to Primitive Streak Formation: A Complementary Normal Table and a New Look at the First Stages of the Development of the Chick

II. Microscopic Anatomy and Cell Population Dynamics

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Received October 31, 1979; accepted in revised form March 24, 1980

The microscopic anatomy of uterine and freshly laid unincubated and briefly incubated chick germs is described. Special attention is paid to the difference between the three developmental periods involved: cleavage, area pellucida formation, and primary hypoblast formation. During cleavage the cytoplasm of the germinal disc divides into blastomeres, which become constantly smaller, and the subgerminal cavity is formed. The germ is accumulating extensive glycogen reserves for utilization during the next period. The most fascinating period is the formation of the area pellucida, which arises as a result of a polarized cell-shedding process. During this process all the subepithelial cells round up and fall into the subblastodermic cavity, where they assemble beneath the future anterior side of the blastoderm. The cell-shedding process is presumably energy consuming and the glycogen reserves are utilized as cell shedding progresses, starting at the posterior and terminating at the anterior side of the germ. The germ loses about one-fifth of its initial cytoplasmic mass during this process. The formation of the primary hypoblast is again polarized, posterioanteriorly. The onset of the process of polyinvagination takes place concomitantly with the shedding of the last subepithelial cells.

INTRODUCTION

In part I of this study the general morphology of the uterine, unincubated and briefly incubated chick embryo was described (Eyal-Giladi and Kochav, 1976). The developmental range from the onset of cleavage to the first appearance of the primitive streak (PS),[2] namely, the period prior to stage 2 H & H (Hamburger and Hamilton, 1951), was divided according to morphological criteria into 14 stages, I–XIV E.G & K (Eyal-Giladi and Kochav, 1976). In addition, a series of morphogenetic processes such as the formation of the subblastodermic cavity, the area pellucida, the primary hypoblast, and the first rudiment of the PS was described and discussed. In the present paper the same stages are dealt with, on the basis of longitudinal sections, and more substantial support is given to the ideas expressed in the first paper. Special attention is paid to a unique process taking place during the formation of the area pellucida, namely, the massive loss of cells, which are shed from the lower layers of the developing germ into the subblastodermic cavity.

MATERIALS AND METHODS

A total of 154 embryos, encompassing the first 13 stages defined by Eyal-Giladi and Kochav (1976), was collected. The embryos of the first 9 stages were extracted from the hen's uterus according to their predicted developmental stage, by pressing the abdomen. Stages X to XIII E.G & K com-

[1] Killed in action, October 16, 1973.

[2] Abbreviations used: PS, primitive streak; c.f., cleavage furrow; c.m., carbon mark; d.c., detached cell; e.y., egg yolk; g.c., glycogen caps; g.p., germ's plasm; h.c., primary hypoblast cells; m.c., middle layer cells; m.f., mitotic figure; m.z., marginal zone; o.c., open cell; s.c., subblastodermic cavity; t.y., tongue containing egg yolk; v.s., ventral spaces; y.s., yolk stumps.

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prised freshly laid unincubated, and embryos incubated for different lengths of time.

The eggs were opened into a bowl containing Ringer's solution. The future posterior side of the germ was marked with carbon according to Von Baer's law (1828) and the germ was then dissected out together with a cushion of underlying yolk in order to preserve properly the relations of germ, subgerminal cavity, and egg yolk. The vitelline membrane was carefully removed and the entire mass fixed with Sunfelice's fluid (Humason, 1967). This fixative was chosen because, in addition to its general quality, it causes the glycogen to aggregate into conspicuous caps at the lower portion of each cell, facilitating the follow-up of glycogen utilization during development (Eyal-Giladi *et al.*, 1979). The germs were embedded in Epon and sectioned at 1-2 μm with an LKB III ultratome. The Epon was removed according to the method of Hoff and Rayburn (1974) and the sections were stained with one of the following: toluidine blue, alcian blue (Humason, 1967), or Lerey and Stahl stain (1961). The sections were then mounted in Picolyte (Turtox).

The data on cell number, cell diameter, blastodermic diameter (the length of the upper contour), and cytoplasmic mass were all calculated from the same median sagittal sections. The upper and lower borders of the blastoderm were drawn as lines following the outer contours of the cells, in the upper and lower layers. Open spaces between lower layer cells were excluded, as well as detached cells in the subblastodermic cavity. Four to eight blastoderms were used for each stage. Counting was done directly from the slide, whereas measurements were done on drawings of sections projected onto a screen. The cytoplasmic mass is represented by the area of the sagittal section and was measured on the drawing with an Amsler planimeter. The mean thickness of the blastodisc was calculated by dividing the area of the cytoplasm by the blastodermic diameter.

RESULTS

Morphology

Period A. Cleavage (stages I–VI E.G & K). Stage I (0–1 hr uterine age; Fig. 1): The cytoplasm of the germ is about 2 mm in diameter (Fig. 21), is relatively poor in both yolk platelets and glycogen, and forms a distinct disc on top of the egg-yolk ball. From the germ's upper surface, one or more vertical cleavage furrows penetrate into the cytoplasmic layer, progressing toward the egg yolk which they have not yet reached. The few giant cells (500–700 μm) are "open" not only on the yolk side but also at their periphery where their cytoplasm is continuous.

Stage II (2 hr uterine age; Fig. 2): Additional vertical furrows appear centrifugally to the earlier ones. At the beginning of the cleavage process there is a slight change in the proportions of the steadily increasing cytoplasmic mass (Fig. 20). The blastodisc becomes slightly thicker and its diameter decreases somewhat (Fig. 21). Tongues of egg yolk are seen to penetrate from below into the cells, which are still open ventrally. Isolated fluid-containing spaces, which tend to expand horizontally, appear underneath the central cells.

Stage III (3–4 hr uterine age; Fig. 3): Cleavage continues both vertically and horizontally. Whereas the peripheral cytoplasm of the germ is not yet cleaved, the blastodisc is two cells thick at the center. The ventral, centrally located, compressed spaces expand horizontally and contain more fluid, but are still isolated from one another. In the central, already closed cells, dark glycogen caps (g.c.) demarcate the lower cell border.

Stage IV (5 hr uterine age; Fig. 4): The vertical cleavage furrows reach the periphery of the germ, horizontal cleaving continues, and, in the center, the blastodisc is three or four layers thick, gradually chang-

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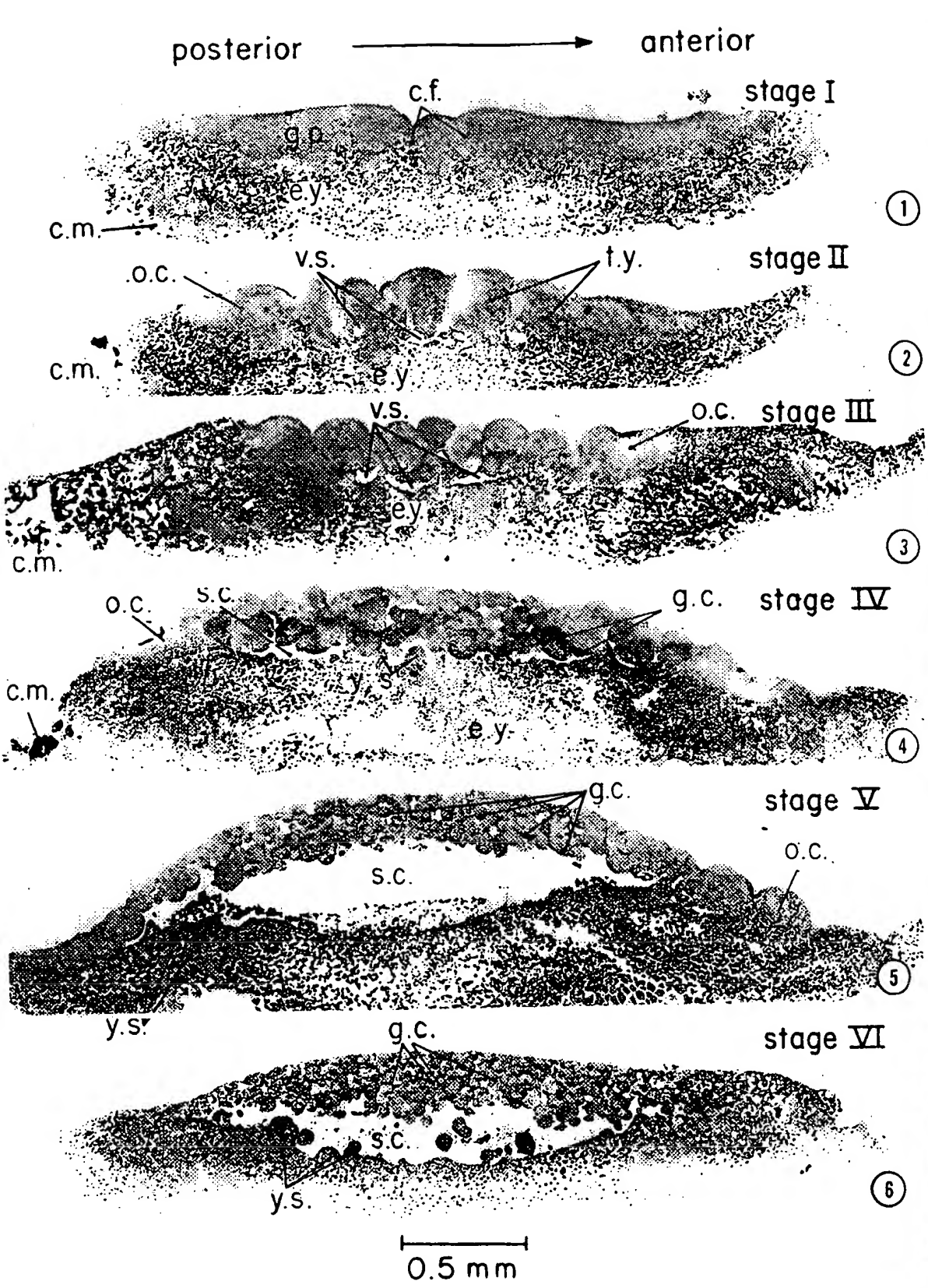

FIGS. 1-13. Median sagittal sections of stages I-XIII E.G & K, respectively.

FIGS. 1-6. Cleavage. Cells become smaller from one stage to the next; starting at stage III the germ becomes multilayered. At stage II, the subblastodermic cavity first appears as small spaces, which enlarge at stage III and fuse to form a continuous narrow cavity at stage IV. At stages V and VI the subblastodermic cavity is expanded. From stage III on, there is a gradual accumulation of glycogen (g.c.), and intracellular yolk platelets become larger and more pronounced.

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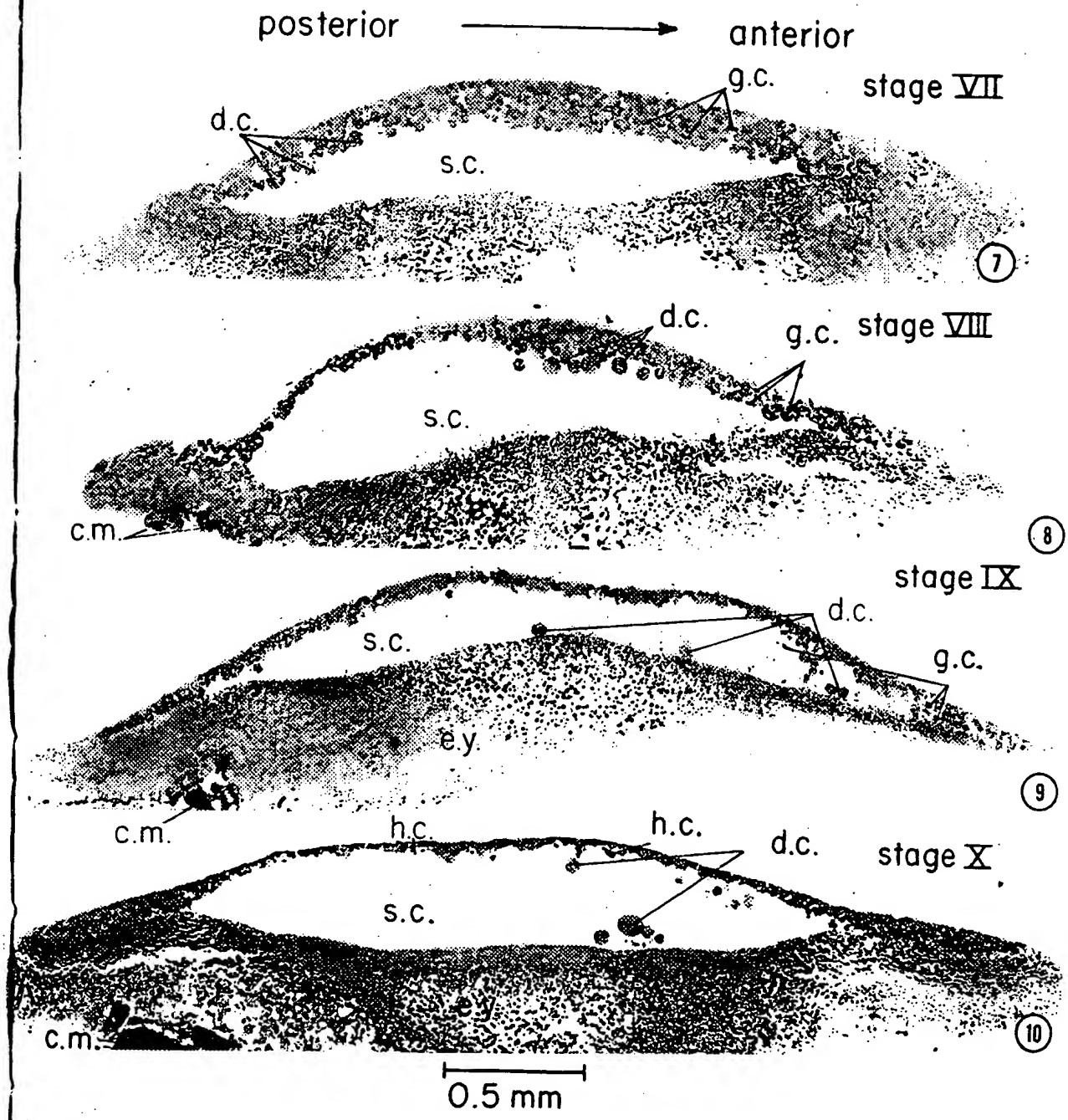

FIGS. 7–10. Formation of the area pellucida. At stage VII the subepithelial posterior cells round up and detach. Glycogen caps disappear from cells of the posterior region but are still pronounced in cells in the anterior three-quarters of the blastodisc. At stage VIII cell shedding reaches the center of the blastodisc and glycogen caps are seen in cells in the anterior quarter only. At stage IX the first wave of cell shedding is almost completed. Glycogen caps are seen in a few anterior cells. At stage X the subepithelial cells persisting after stage IX have been disposed of and islands of polyinvaginating primary hypoblastic cells appear beneath blastodermic epithelium, now one cell layer in thickness.

ing to one or two cells thick toward the periphery. The most peripheral cells are still open cells (o.c.). Underneath the central part of the germ, there is a narrow continuous subblastodermic cavity (s.c.). The irregular ventral face of this cavity indicates its formation by fusion of the many separate ventral spaces. Most of the

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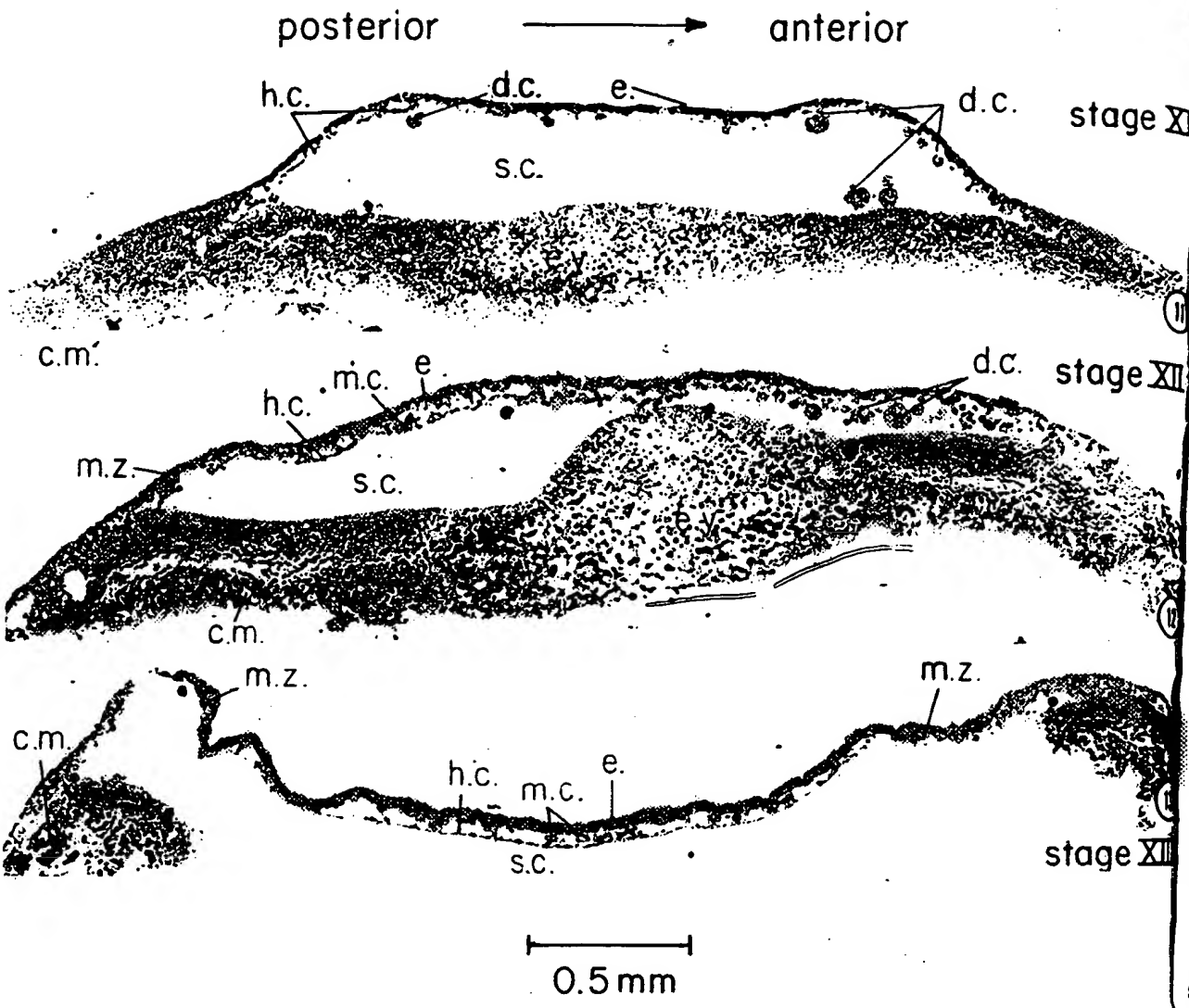

FIGS. 11-13. Formation of primary hypoblast. At stage XI several posterior fragmentary sheets of primary hypoblast cells are seen. Anterior to them, there are scattered hypoblast cells. Detached yolk-laden cells are found in the subblastodermic cavity. At stage XII the primary hypoblast forms a continuous sheet covering the lower surface of the posterior half of the blastoderm. Many detached yolk-laden cells are found inside the subblastodermic pocket. At stage XIII primary hypoblast covers the entire central area of the blastoderm. Peripheral to it is the ring-like marginal zone not covered by the hypoblast. Clusters of middle layer cells between epiblast and hypoblast.

cells of the blastodisc have ventral glycogen caps.

Stage V (8-9 hr uterine age; Fig. 5): Cleavage continues in all directions and the cells become smaller. At the periphery there are still big open cells. The main change is the formation of a spacious subblastodermic cavity, probably resulting from accumulation of fluid. The glycogen caps are more distinct and large yolk platelets are more pronounced.

Stage VI (10-11 hr uterine age; Figs. 6 and 14): There are no longer large open cells at the periphery. At its center the blastodisc is four or five cells thick. The glycogen caps are very prominent. The cells, especially the deeper cells, are full of large yolk platelets. The yolk-side face of the subblastodermic cavity is still wavy, with stumps protruding from it into the cavity (y.s.).

Period B. Area pellucida formation

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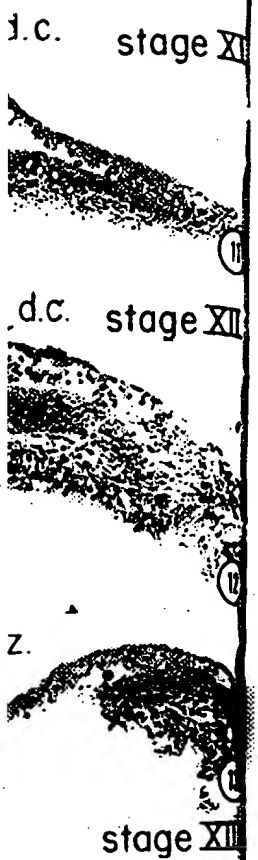

(stages VII–X E.G & K). Stage VII (12–14 hr uterine age; Figs. 7 and 15): The sagittal section, which until this stage did not show a remarkable difference between its anterior and posterior ends, exhibits for the first time a clear anterioposterior polarity. While the cells at the center and the anterior end continue cleaving and become still smaller, the cells at the deeper layers of the posterior end round up, detach from the blastodisc (d.c.), and fall into the subblastodermic cavity. As a result of this, the posterior part of the blastodisc gets thinner and more transparent and the area pellucida starts to appear at the future posterior side of the germ. The glycogen caps characteristically disappear from the region involved in cell shedding, whereas they are still prominent in cells at the more anterior parts of the germ. The subblastodermic cavity is somewhat more expanded and its ventral surface has become smooth.

Stage VIII (15–17 hr uterine age; Figs. 8 and 16): The process of cell shedding continues in an anterior direction and reaches about the center of the germ. The shed cells are large and rounded and contain many yolk granules. The glycogen caps have disappeared from most cells and are seen only in cells of the anterior third of the germ.

Stage IX (17–19 hr uterine age; Figs. 9 and 17): The first massive wave of cell shedding is almost completed. The germ is one to three cells thick, a few cell clusters are still attached to its lower surface, and in these cells glycogen caps are still visible. Free yolk-laden cells are seen in the subblastodermic cavity.

Stage X (a freshly laid egg, about 20 hr uterine age; Figs. 10 and 18): All the cells of the lower layer of earlier stages have now been shed, and some are seen in the cavity. The uppermost layer of the germ has developed into an organized epithelium and a new generation of small cells, appearing at many foci, is aligning itself into small isolated epithelial sheets, more abundant at the posterior than at the anterior end of the blastoderm.

Period C. Hypoblast formation (stages XI–XIII E.G & K). Stage XI (Figs. 11 and 19): The blastoderm becomes a cylindrical epithelium with about one-third of its posterior area lined with large fragments of primary hypoblastic sheets. Beneath the anterior part of the blastoderm, only isolated clusters of hypoblastic cells can be seen. Most of the shed cells remain in the cavity underneath the forming primary hypoblast, and only occasionally is such a cell located between the epiblast and the hypoblast or within the hypoblast itself.

Stage XII (Fig. 12): The primary hypoblast covers the posterior half of the ventral surface of the blastoderm, forming an almost continuous flat epithelial sheet.

Stage XIII (Fig. 13): The primary hypoblast is completed and covers a large central area of the ventral side of the epiblast, the hypoblast-free ring of the area pellucida being the marginal zone (m.z.) of the blastoderm. Between the epiblast and the hypoblast, scattered middle layer cells (m.c.) already exist prior to the formation of the primitive streak.

Quantitative

Cell number (Fig. 20). Total cell number per median sagittal section (Fig. 20A) increases slowly during the first three stages to about 20. Between stages III and VII there is a dramatic increase to about 460 cells per section. From stages VII to IX the total number continues to increase moderately, and from stages IX to X one-fifth of the total number of cells disappears from the blastodisc. From stage X onward cell number increases again. The picture changes when the total cell number is dissociated into two components, the upper-layer cells (epithelial) and all the other cells, which we refer to as subepithelial cells. The number of epithelial cells (Fig. 20C) increases steadily throughout the stages studied; the subepithelial cells (Fig.

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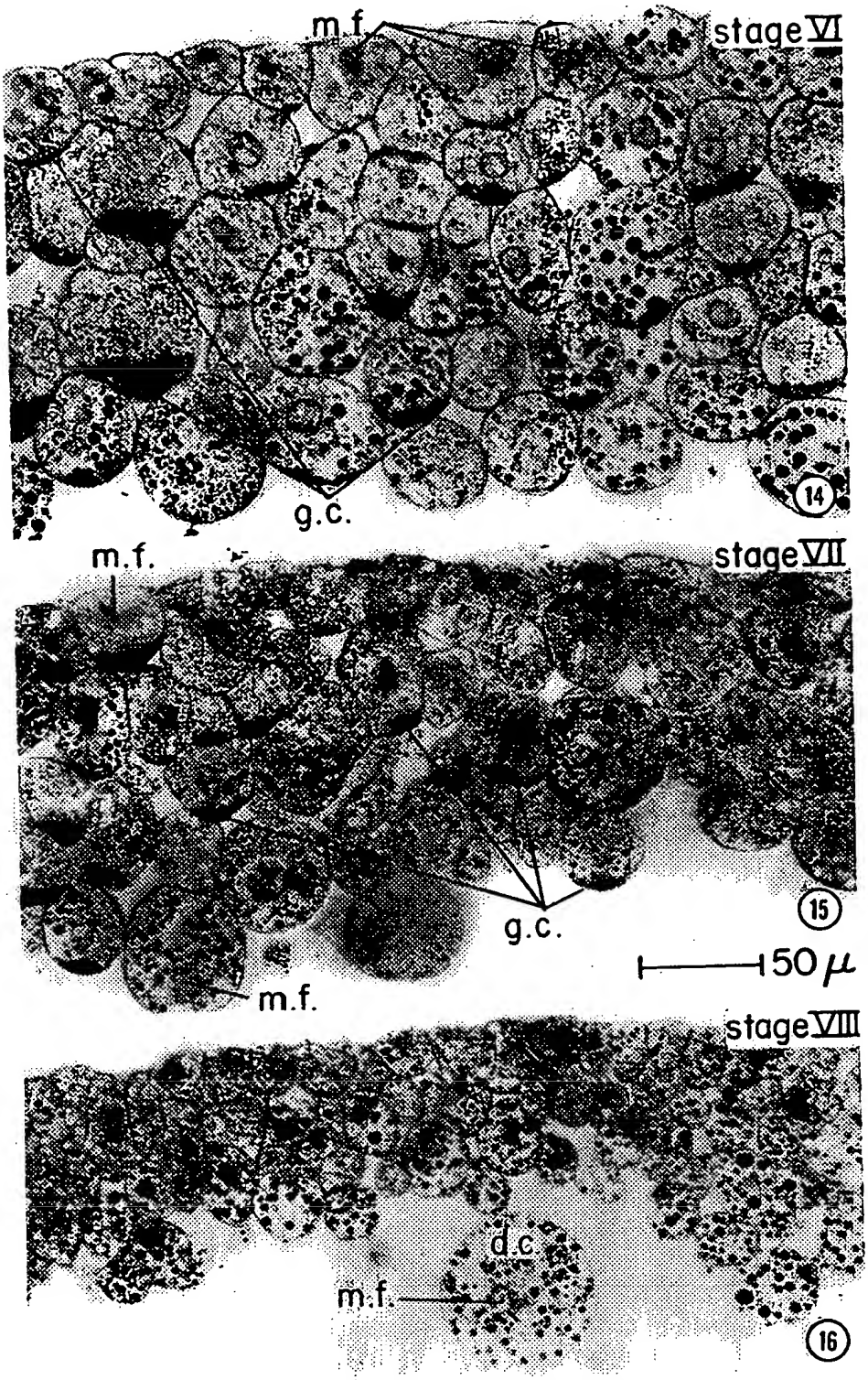

FIGS. 14–19. Magnifications of central section of germs at stages VI–XI, respectively.

20D), which appear for the first time at stage III, increase very rapidly up to stage VII. From stages VII to X there is a 46% net loss in cell number, which is rather slight (5%) for stages VII and VIII and qui dramatic (41%) from stages IX to X. A stage XI there is already a renewed increas in the number of subepithelial cells.

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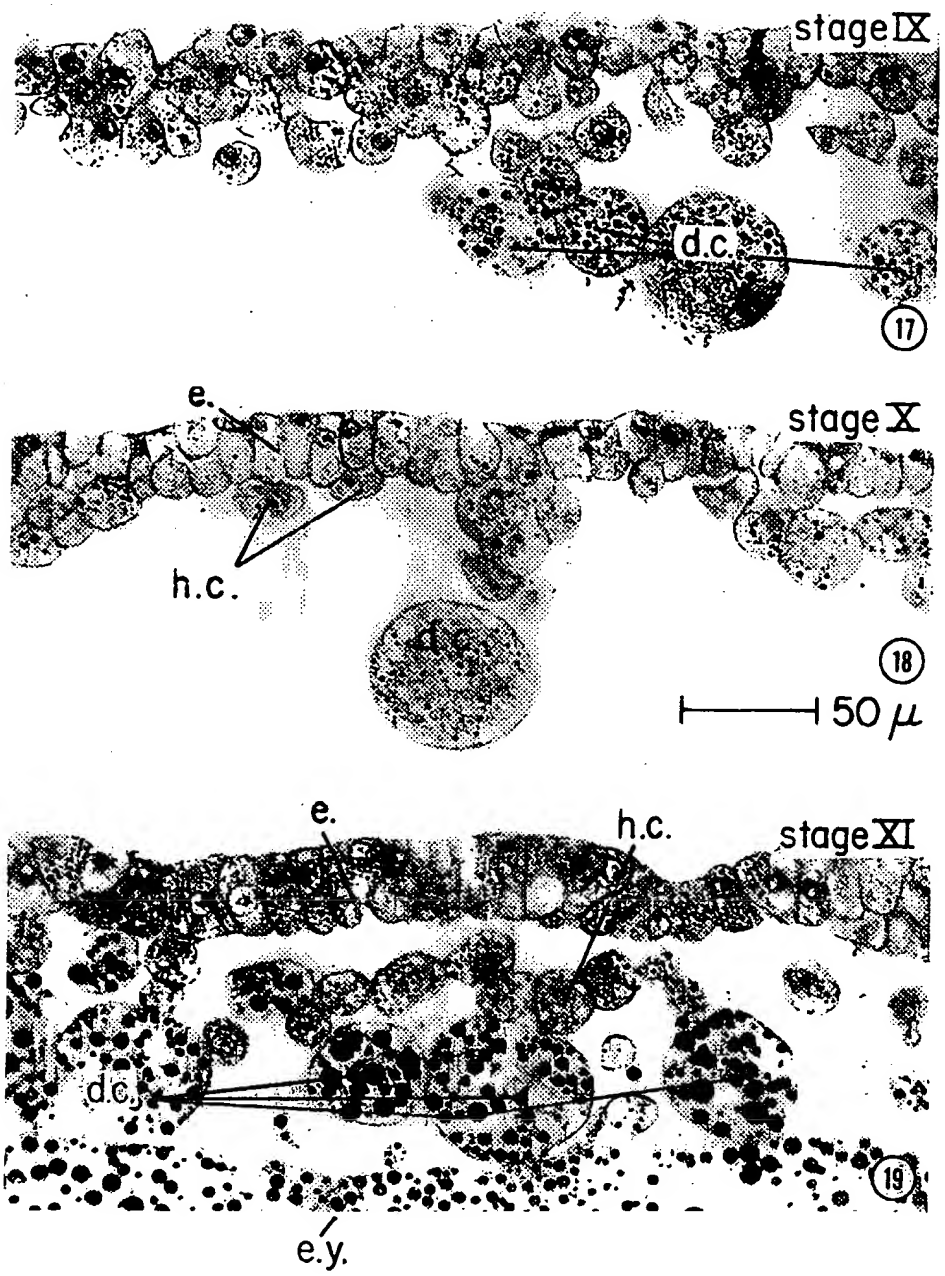

Cytoplasmic mass (Fig. 20A). The entire area of the median sagittal section of the blastodisc (disregarding the cellular borders) was measured and used as an indicator of its total cytoplasmic mass. The cytoplasmic mass grows from stages I to IV, after which it remains constant up to stage VII. Between stages VII and X the blastodisc loses three-quarters of its mass. After stage X the blastoderm gradually starts to regain cytoplasmic mass.

Cell diameter (Fig. 21). In general, cell diameter is heterogeneous even in a given sagittal section. Cells are usually smallest in the upper layer and become larger the deeper they are situated, with the largest cells in the lowest layer. In any given layer the central cells are the smallest and cells gradually increase in size toward the periphery. It is therefore impossible to present an average diameter for the cells during the stages considered in the present study. However, in order to provide some indication of the overall changes in cell size, the

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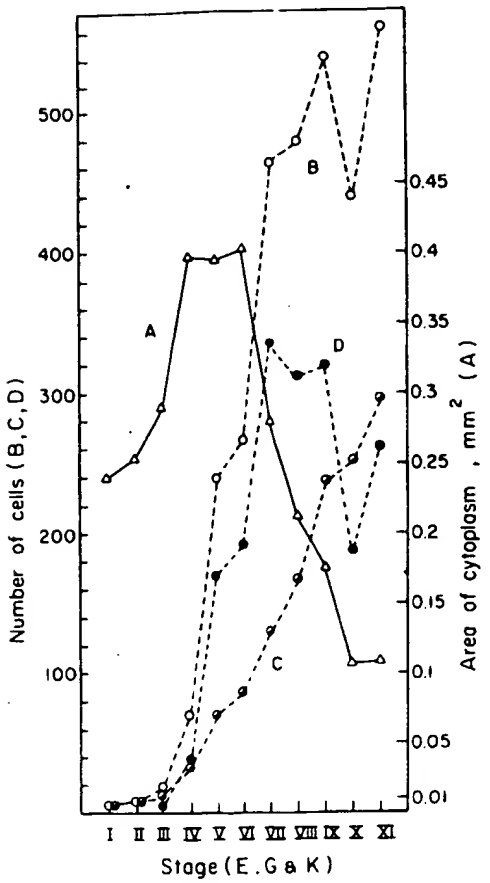

FIG. 20. (A) Average area of cytoplasm (mm^2); (B) total cell number; (C) cell number in upper epithelial layer; (D) cell number in subepithelial layers of germs at the various stages.

mean diameter was calculated for the uppermost layer only (all the cells which are included in the upper surface), by dividing the diameter of the blastoderm by the number of cells counted in the upper layer. Up to stage V the mean diameter is rapidly reduced. From stage VI on, the reduction in cell diameter becomes much more gradual.

Diameter of the germ (Fig. 21A). The diameter of the germ at the first three stages is about 2 mm. However, there is a tendency to reduction from stages I to II. After stage III there is a steady increase in diameter.

Thickness of the germ (Fig. 21B). This was calculated by dividing the mean cytoplasmic area for each stage by the mean diameter of the same blastoderms. There is a thickening of the germ between stages I and II, after which the thickness remains constant at about 150 μm up to stage VII. From stages VII to X there is a drastic thinning of the germ to an average value of about 30 μm, one-fifth of that before the onset of thinning.

DISCUSSION

The morphology of the blastoderm from the beginning of cleavage to the appearance of the primitive streak was studied by Eyal-Giladi and Kochav (1976). Fourteen developmental stages have been defined, covering development prior to stage 2 H & H. The observations of Eyal-Giladi and Kochav were based on live material and were made with a stereoscopic microscope. Three distinct developmental periods were described, each characterized by a specific developmental process. The period between stages I and VI, during which the subgerminal cavity is also formed, was defined as cleavage per se. Stages VII–X were

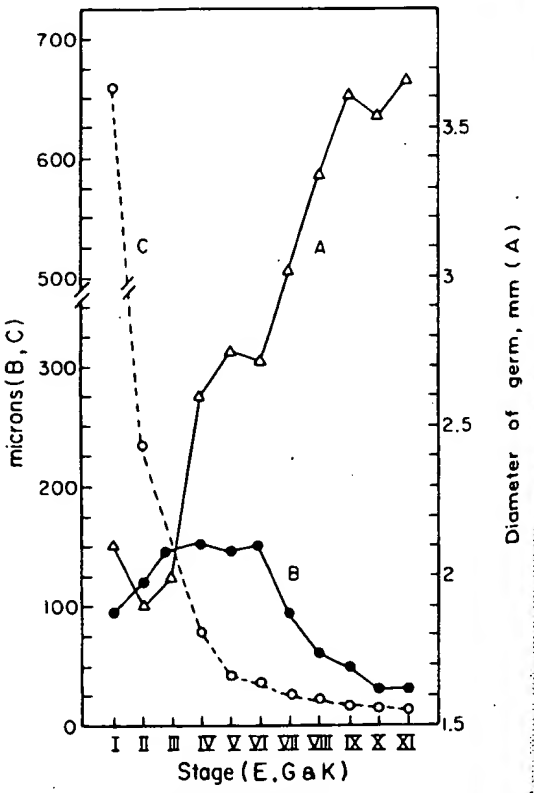

FIG. 21. (A) Average diameter (mm); (B) thickness of germs (μm); (C) diameter of upper layer of cells at the various stages.

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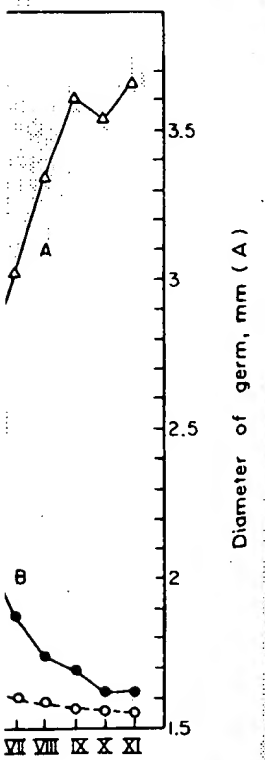
characterized by the oriented formation of the area pellucida from posterior to anterior by a process of cell shedding, and stages XI–XIV were characterized by the primary hypoblast formation according to the same posterioanterior polarity. In the present study, which is based on light microscopy, we followed more explicitly the processes described previously.

Cleavage (Stages I–VI)

Eyal-Giladi and Kochav (1976) discussed the various views and the resulting confusion concerning the formation of the subgerminal cavity and the formation of the area pellucida. Their findings supported observations of His (1868) and Goette (1874) that the subgerminal cavity is a real cavity gradually formed by the accumulation of fluid. His specifically mentioned the accumulation of "vacuoles" underneath the germ that gradually contribute to the formation of the cavity. By observing live germs, we noticed the existence of abundant vacuoles, however, we could not state conclusively that the vacuoles contribute to the subgerminal cavity. In the present study, care was taken to fix the germ supported by a thick layer of yolk, so as to preserve as much as possible the normal relations between the germ, cavity, and underlying yolk. The observations have confirmed His' (1868) conclusion. Isolated fluid-containing spaces, which appear in the borderline between the germ plasm and the egg yolk phase as early as stage II, grow in size and spread from the center toward the periphery, fusing with one another. At stage IV the subgerminal cavity is already formed, but its contours indicate the mode of its formation. There is relatively little subgerminal fluid in it, accumulation of fluid seeming to occur very rapidly between stages IV and VI.

The blastodisc during cleavage (stages I–VI) may be examined from two angles. Regarding the cell population, there is a constant and impressive increase in total cell number (Fig. 20B) and, at the same time, a drastic reduction in cell diameter (Fig. 21C), both phenomena fitting the definition of cleavage. Looking separately at the upper epithelial layer and the subepithelial cells, the same trend for a remarkable increase in cell number during cleavage is seen. However, this increase is more pronounced in the subepithelial category. We assume that this might be an indication not of a higher rate of division in the subepithelial cell fraction, but rather that, in addition to division of the subepithelial cells, additional cells are descending from the dividing epithelial layer into the subepithelium. Although there are morphologic indications for such a process, this suggestion needs to be confirmed by an accurate study of the mitotic activity at the different developmental stages. This study is now underway.

Another way of looking at the cleaving blastodisc is to regard it as an entity in itself. Here the picture exhibits different dynamics. Looking at the diameter of the blastodisc (Fig. 21A), an initial decrease is detected, namely, the blastodisc appears to be getting smaller, but the observation is obviously deceptive, for at the same time it is also getting thicker, and in fact there is a small net increase in cytoplasmic mass as represented by the mean cytoplasmic area of the sections (Fig. 20A). Between stages III and IV there is a remarkable growth of the cytoplasmic mass, but the thickness of the blastodisc remains constant, while it increases in diameter. From a histological point of view, there is a clear difference between the blastodiscs of stage III (Fig. 3) and stage IV (Fig. 4), the latter being richer in large intracellular yolk platelets and stored glycogen (see Eyal-Giladi *et al.*, 1979). The appearance of these reserve materials probably indicates an enhanced metabolic activity. The two following stages, V and VI, exhibit a remarkable slowing down of growth, as the cytoplasmic area and the thickness of the blastodiscs remain con-



meter (mm); (B) thickne
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stant, and probably also the blastodiscs' diameters. The difference in diameters between stages IV and VI (Fig. 21A) is negligible ($P = 0.31$) compared to the differences between diameters at stages III and IV ($P = 0.01$) and stages VI and VII ($P = 0.002$), which are significant. The temporary slowing down of growth at stages IV–VI seems to be preparatory to the approaching morphogenetic event starting at stage VII.

Formation of the Area Pellucida (Stages VII–X)

The microscopic sections confirm our previous observations (Eyal-Giladi and Kochav, 1976). The formation of the area pellucida is the result of a massive cell loss. At stage VII (Figs. 7 and 15) the lowest cells of the subepithelial layer start rounding off at the future posterior side of the blastodisc, thereby giving the first clear indication of the polarization of the germ during the previous developmental period (Kochav and Eyal-Giladi, 1971; Eyal-Giladi and Fabian, 1980). The rounding off and cell shedding proceed in a posterioanterior direction. At stage VIII (Fig. 8) they reach approximately the middle of the germ, and at stage IX the anterior end (Fig. 9). However, not the entire thickness of the subepithelial layer is involved in this process right from the beginning, so that during the first wave of cell shedding, only a layer about two or three cells thick is shed (Figs. 9, 15, 16, and 17), the rest being shed only at stages IX–X, leaving the germ with an epiblast mainly one cell thick (Figs. 18 and 19).

The oriented morphogenetic process of the formation of the area pellucida is clearly correlated with a speedy utilization of the glycogen reserves which have been accumulating in the cells during cleavage (Eyal-Giladi *et al.*, 1979). The degradation of glycogen does not proceed equally in all cells through stages VII–IX, but is also a polarized process. The glycogen caps begin to disappear at stage VII in the posterior region (Fig. 7); they later disappear also from the central region (Fig. 8), and at stage IX (Fig. 9) only a few anterior cells contain glycogen caps. By stage X (Fig. 10) there are no longer any glycogen caps. This may indicate that cell shedding and the formation of the area pellucida are energy-consuming processes. The beginning of cell shedding at stage VII is not yet reflected by the total cell number (Fig. 20B), of which there is an impressive increase compared with stage VI. This increase is probably due to the high rate of cell divisions in both the epithelial and the subepithelial parts of the blastodisc. Between stages VII and VIII there is a turning point, and although the total number of cells continues to rise moderately, there is a significant difference between the two cell categories. Whereas the number of epithelial cells continues to grow almost linearly as before, the number of subepithelial cells shows the first indication of reduction. This occurs despite the fact that the subepithelial cells also continue to divide (Fig. 15). The same phenomenon is true for the period between stages VII and VIII, and stages VIII and IX, where even detached cells show mitotic figures (Fig. 16). Between stages IX and X cell shedding becomes so pronounced that the total cell number decreases. Nevertheless, the cell number in the upper layer continues to rise, and the entire loss has to be accounted for by the remarkable reduction in the number of subepithelial cells. The reason Fig. 20D indicates that relatively many subepithelial cells seem to have remained as late as stage X is that the cells of the marginal zone and area opaca have also been included, and most of the cells of the subepithelial category are located in those regions and not in the area pellucida proper.

Regarding the blastodisc as a whole, after the relatively quiescent period between stages IV and VI, the diameter increases remarkably between stages VI and X (Fig. 21). The other parameters, however, reflect more closely the morphogenetic process of cell shedding, and from stage VI to X the thickness of the blastoderm is reduced to about one-fifth. The cytoplasmic mass

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drops at stage X to about a quarter of the mass at stage VI. We believe that the preceding analysis of our data does not leave any doubt that during the formation of the area pellucida, the period of symmetrization, the blastodisc is in the process of losing most of its cellular cytoplasmic mass.

The phenomenon of such an organized enormous loss of cells during the early stages of embryogenesis is thus far unique, the meaning of which we do not yet understand. The role of the detached cells is also still an enigma. We know that because of the oblique position of the germ inside the rotating egg in the mother's uterus (Kochav and Eyal-Giladi, 1971), all these round cells [Dotter-Kugeln of Goette (1874), Peter (1938), or Furchungskugeln of Kölliker (1875)] roll within the subgerminal cavity to the lowest point, which is the future anterior side of the blastoderm (Figs. 10, 11, and 12). The anterior position of these "yolk balls" can be verified also in live material when care is taken not to interfere with the spatial position of the germ. How such a considerable concentration of large loose cells affects the developing blastoderm is still unclear. However, it is interesting to stress that this concentration is on the side where events do *not* take place (anterior side) and that all the morphogenetic processes begin at the point farthest from it. It is therefore possible that these cells might have an inhibitory effect on developmental processes.

The issue of primary hypoblast formation has already been examined by Eyal-Giladi and Kochav (1976), and the present observations corroborate their conclusions. Concomitantly with the shedding of the last subepithelial yolk laden cells, the process of polyinvagination of clusters of much smaller hypoblastic cells begins (Fig. 18). The newly formed hypoblastic cells are relatively more crowded at the future posterior side of the blastoderm, where they also begin to form the hypoblastic layer, a morphogenetic process which spreads in a posterioanterior direction. In our material it was impossible to distinguish between different types of primary hypoblastic cells. However, it should be borne in mind that the polyinvaginating cells probably do not possess the capacity to induce a primitive streak. The latter role is confined to primary hypoblastic cells, derived from the marginal zone (Azar and Eyal-Giladi, 1979), which grows inward to merge with polyinvaginated cells.

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